

AD_____

Award Number: DAMD17-01-1-0506

TITLE: Exploiting Polytene Chromosomes to Identify Transcription
Cofactors and Complexes Recruited to an Estrogen Responsive
Promoter
In Vivo

PRINCIPAL INVESTIGATOR: Steven K. Nordeen, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado Health Science
Center
Denver, Colorado 80045-0508

REPORT DATE: June 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

20020913 003

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE June 2002	3. REPORT TYPE AND DATES COVERED Final (15 May 2001 - 14 May 2002)	
4. TITLE AND SUBTITLE Exploiting Polytene Chromosomes to Identify Transcription Cofactors and Complexes Recruited to an Estrogen Responsive Promoter In Vivo			5. FUNDING NUMMER DAMD17-01-1-0506	
6. AUTHOR(S) Steven K. Nordeen, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Colorado Health Science Center Denver, Colorado 80045-0508 email - steve.nordeen@uchsc.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Report contains color				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) The purpose of this work was to develop a novel system to study the recruitment of factors, especially those involved in chromatin modification and remodeling, to an estrogen-responsive promoter. To do this, transgenic fruitflies were created that conditionally express estrogen receptors and that contain a reporter whose expression is dependent on the estrogen receptor. Six doubly transgenic lines were developed. These transgenic flies were shown to express the estrogen receptor in response to heat shock and the reporter (green fluorescent protein) in response to estrogens. For two of the lines thus far, estrogen-dependent expression of the reporter in salivary glands has been confirmed. We have also shown that estrogen receptor moves from regions of the nucleus not occupied by chromatin to chromatin upon activation of the estrogen receptor by hormone agonists. We are now preparing to assess hormone-dependent recruitment of factors to the target locus by fluorescence immunohistochemistry of chromosome squashes. In this way we will be able to assemble a detailed picture of the functional complexes that mediate estrogen action and delineate the time course of their formation. In particular, this will lead to a better understanding of the role of chromatin remodeling and modification in estrogen action.				
14. SUBJECT TERMS breast cancer, polytene chromosomes, transcription cofactors in vivo				15. NUMBER OF PAGES 9
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	5
Conclusions.....	6
References.....	
Appendices.....	7, 8, 9

INTRODUCTION

This research project was supported by a Concept Award and represents the work stemming from one year of effort. The aim of the research project was to develop a novel system for the elucidation of molecular details of the action of estrogen receptor with a particular focus on delineation of the timing and nature of the factors that are recruited to an estrogen regulated promoter. To achieve this it was proposed to create lines of transgenic fruitflies with a reporter gene regulated by estrogen response elements and that expressed estrogen receptor. The concept was to take advantage of the endoduplication that results in the polytene chromosomes characteristic of specific tissues such as the salivary gland of the developing fly. Once these double transgenic lines were developed, each had to be verified for the ability to respond to estrogens, particularly in the salivary gland. The binding of factors to specific chromosomal sites can be directly visualized by straightforward immunohistochemical techniques on chromosomal squashes from salivary glands. The ultimate goal of this research is to delineate a detailed kinetic and compositional analysis of the events that occur at an estrogen-responsive gene following the binding of the hormone-bound estrogen receptor to the promoter of a target gene.

BODY

As a Concept Award the proposal had no formal Statement of Work. The research accomplishments that addressed to goals outlined in the proposal will be addressed. At the time the proposal was written we had developed lines of transgenic fruitflies that contained sequences encoding the estrogen receptor (ER) under the control of the heat shock 70 promoter and had mapped the chromosomal locations of the transgenes. We had also developed lines of flies that contained a reporter gene (green fluorescent protein, GFP) under the control of a promoter regulated by estrogen response elements (EREs) and had mapped their chromosomal locations. Our initial efforts funded by this award were to demonstrate that the ER-transgenic flies could indeed express ER following heat shock and to optimize the heat shock and recovery conditions. An example of estrogen receptor expression in salivary glands from heat shocked larvae is shown in Figure 1A. Receptor staining is confined to the nucleus. The parental line of flies from which the transgenic flies are derived (yellow white, YW) exhibit no staining under the same conditions (Figure 1B). Two lines of flies, 1.3 and 3.2, that expressed ER robustly in response to a 90 min incubation of the larvae at 37C (heat shock conditions for fruitflies) were selected. These lines contain the ER gene on different chromosomes (II and III, respectively). These lines were bred to appropriate balancer lines as were the lines containing the reporter genes. Line 1.3 was then crossed to lines 11.1 and 11.2 containing the reporter gene on a different chromosome than the ER gene. This was done to prevent any possible interference between the loci when it came time to do the staining of chromosome squashes. ER line 3.2 was crossed to four reporter lines where the reporter is again on a different chromosome from the ER. In total then we have 6 doubly transgenic lines with a heat shock regulated ER gene and a ERE regulated GFP reporter gene. The properties of these are shown in the table included with the Reportable Outcomes section.

Once the doubly transgenic lines were developed we assessed whether the lines were, in fact, responsive to estrogens. Larvae for each of the 6 lines were grown on medium with or without estrogen. Larvae from estrogen-fed and control vials were heat shocked for 90 min at 37C. Another set of estrogen-fed larvae were not heat shocked. Larvae were then allowed to recover for various times and GFP expression was assessed by fluorescence microscopy. Heat shocked larvae grown on estrogen

medium exhibit GFP expression whereas heat shocked flies grown on control medium exhibited only the autofluorescence typical of fruitfly larvae. Flies that were not heat shocked to increase ER expression but were grown on estrogen-containing medium occasionally exhibit a small amount of GFP fluorescence. This is likely due to a small basal level of ER expression from the heat shock 70 promoter. Figure two shows fluorescence of three larvae. The top one is heat shocked, grown on control medium. The middle one is heat shocked, grown on estrogen medium, the bottom larva was not heat shocked but grown on estrogen medium. Only the middle larva exhibits substantial GFP expression establishing that GFP expression is dependent on hormone and expression of ER. We have determined that all 6 double transgenic lines express GFP in a receptor- and hormone-dependent manner.

Not all tissues expressed GFP equally well in response to estrogens. Also, although all lines express GFP in response to estrogen, not all do in the same pattern or to the same degree. Therefore, we felt it necessary to assess hormone-dependent GFP expression in salivary glands. Thus far we have confirmed for two lines that GFP is expressed in salivary glands appropriately by dissecting glands from larvae grown on control and estrogen medium. See table in Reportable Outcomes. Experiments are currently underway to assess GFP expression in the other 4 cell lines.

In another set of experiments salivary glands have been dissected from 3rd instar larvae that had been heat shocked and allowed to recover and express ER for 4 hours. The glands were placed in medium containing an estrogen for 30 minutes. The tissues were fixed and prepared for immunostaining. High resolution fluorescence microscopy reveals that estrogen receptor is nuclear but not associated with chromosomes in the absence of hormone treatment. Exposure to an estrogen changes the localization of receptor. It is now specifically associated with chromatin.

Finally, arrangement have been made for the P.I. to spend a month in the laboratory of John Lis at Cornell University in order to learn chromosome squash techniques and to do some initial high resolution staining to look at recruitment of factors to the estrogen-dependent gene locus. This visit will take place for the month of July, 2002.

KEY RESEARCH ACCOMPLISHMENTS

- Developed immunohistochemical and Western blot methodologies for detection of estrogen receptor (ER) in fruitfly larvae and dissected salivary glands.
- Screened hsp70:ER transgenic flies for those lines that have highest expression of ER.
- Optimized heat shock conditions to achieve high ER expression.
- Selected two lines, 1.3 and 3.2, for crossing to transgenic fly strains containing reporter genes under the control of estrogen response elements.
- Crossed ER transgenic lines with 6 independent, mapped, reporter lines.
- Assayed all six double transgenic lines to confirm that the reporter gene, green fluorescent protein (GFP) is expressed in larvae in response to heat shock (to induce estrogen receptor) and an estrogenic hormone.
- Confirmed hormone-dependent expression of GFP in salivary glands dissected from larvae derived from 2 of the six strains.
- Performed immunohistochemical detection and fluorescence microscopy to determine that ER from salivary glands not treated with hormone is nuclear but not associated with chromatin, whereas ER in hormone-treated glands is associated with chromatin.

- Made arrangements to visit the laboratory of John Lis at Cornell University to learn chromosome squash technology in order to assess the recruitment of factors to the estrogen-responsive reporter genes. The visit will occur in July 2002.

REPORTABLE OUTCOMES

This work will be presented at the ERA of Hope meeting in September 2002. A copy of the abstract has been appended.

The following 6 strains of transgenic fruitflies that are responsive to estrogens have been developed:

Strain name	Chromosomal Location of ER Transgene	Chromosomal Location of GFP Reporter Gene	Confirm express GFP	
			Larvae	Salivary gland
1.3/11.1	II	III	+	nt*
1.3/11.2	II	III	+	+
3.2/10.5	III	II	+	nt
3.2/10.6	III	II	+	+
3.2/4.2	III	X	+	nt
3.2/8.1	III	X	+	nt

*nt = not tested

CONCLUSIONS

We have completed the development of an estrogen-responsive reporter system that can now be exploited to examine the estrogen receptor-dependent recruitment of chromatin remodeling and modification factors as well as components of the basal transcription complex. Moreover, we are poised to be able to ask detailed questions about the kinetics of the recruitment of these different factors. This is a powerful system that can be used to address questions on the molecular mechanisms of estrogen receptor action that are much more difficult or impossible to do in mammalian systems.

Appendix

Abstract to be presented at the Era of Hope meeting

Curing Breast Cancer in Fruitflies. What are you nuts?!

Steven K. Nordeen, Richard Heyer, Joan Hooper University of Colorado Health Sciences Center, Denver, CO 80262

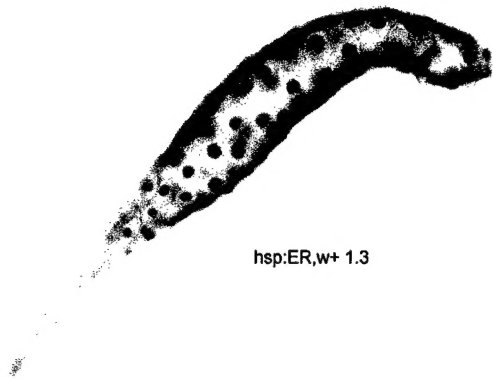
Estrogens play a critical role in the development and growth of the mammary gland. Estrogen antagonist therapy is widely used in the treatment of estrogen-dependent tumors and as a prophylactic in women at high risk for breast cancer. Rational intervention with estrogen signaling is handicapped by the limited understanding of the molecular mechanisms involved. In order to understand the regulatory mechanisms of the estrogen receptor, it is critical to elucidate the factors that play a role in ER-dependent transcription. The specific goal of this Concept Award is to elucidate the nature and timing of the factors recruited to estrogen responsive promoters in response to hormone. To do so, a novel approach has been developed that exploits properties of the chromosomes of the fruitfly, *Drosophila melanogaster*. The polytene chromosomes of the salivary gland permit direct immunocytochemical detection and localization of factors associating with distinct sites on chromatin. We have engineered several lines of flies that conditionally express ER under the control of the heat shock promoter and additional lines of transgenic flies with ERE-containing transgenes at different chromosomal sites. These lines have been crossed to produce doubly transgenic flies with both the ER and the ER-responsive transgenes. Currently we are assessing hormone-regulation of the transgenes in the different strains. Once expression is confirmed we will perform in situ hybridization on chromosomal spreads to visualize the location of the transgenes in different lines. The amplification and loose packing of non-centromeric chromatin in the polytene chromosomes will allow direct visualization of the recruitment of factors to the transgenes including the receptor itself. We will then assess the recruitment of a variety of factors that are involved or suspected to be involved in steroid receptor-mediated transcription including RNA polymerase II, TATA binding protein, chromatin remodeling factors such as Brahma, Acf1, and ISW1, histone acetyltransferases, and other putative coactivators by immunohistochemical detection using antibodies to the *Drosophila* versions of these factors. In addition we will assess the state of histone modification at the promoter. The results of these studies will be presented. These studies will help to provide the fundamental information necessary if we are to understand the subtleties of estrogen receptor action necessary to fully exploit hormone-based therapies for breast cancer.

Figure Legends

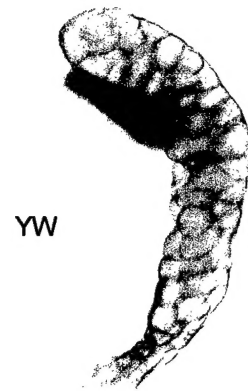
Figure 1. Expression and cellular localization of estrogen receptor in the salivary gland of transgenic fruitflies. A. Larvae of transgenic fly strain 1.3 were subjected to a 90minute heat shock at 37C then allowed to recover for 4 hours at room temperature to express the estrogen receptor. Salivary glands were dissected, fixed, and stained for human estrogen receptor. B. Same as A except non-transgenic flies were used.

Figure 2. Expression of GFP in a hormone- and receptor-dependent fashion in 3rd instar larvae of doubly transgenic flies. Three larvae are shown in the field of the dissecting fluorescence microscope. The top larva was heat shocked to induce estrogen receptor expression and grown on control medium. It exhibits only dim autofluorescence. The middle larva was heat shocked and grown on medium containing an estrogen. The flies were grown overnight following heat shock. The bottom larva was also grown on medium containing estrogen but was not heat shocked. Estrogen receptor expression in this line is quite low.

1A



1B



2

